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The Expanding Clinical Phenotype of the tRNA$^{Leu(UUR)}$ A→G Mutation at np 3243 of Mitochondrial DNA: Diabetic Embryopathy Associated With Mitochondrial Cytopathy

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We describe a family which demonstrates and expands the extreme clinical variabilty now known to be associated with the A→G transition at nucleotide position 3243 of the mitochondrial DNA. The propositus presented at birth with clinical manifestations consistent with diabetic embryopathy including anal atresia, caudal dysgenesis, and multicystic dysplastic kidneys. His co-twin was normal at birth, but at 3 months of life, presented with intractable seizures later associated with developmental delay. The twins' mother developed diabetes mellitus type I at the age of 20 years and gastrointestinal problems at 22 years. Since age 19 years, the maternal aunt has had recurrent strokes, seizures, mental deterioration and deafness, later diagnosed as MELAS syndrome due to the tRNA$^{Leu(UUR)}$ A→G mutation. A maternal uncle had diabetes mellitus type I, deafness, and normal intellect, and died at 35 years after recurrent strokes. This pedigree expands the known clinical phenotype associated with tRNA$^{Leu(UUR)}$ A→G mutation and raises the possibility that, in some cases, diabetic embryopathy may be due to a mitochondrial cytopathy that affects both the mother's pancreas (and results in diabetes mellitus and the metabolic dysfunction associated with it) and the embryonic/fetal and placental tissues which make the embryo more vulnerable to this insult.

KEY WORDS: mitochondria, MELAS, diabetic embryopathy, caudal regression, anal atresia, diabetes mellitus, seizures

INTRODUCTION

In 1990, the A→G transition at position 3243 of the mitochondrial DNA (mtDNA) was first described to be the pathological mutation in 80% of cases [Goto et al., 1990] with MELAS (mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes) syndrome (McKusick 590050.0001) [Pavlakis et al., 1984; Koo et al., 1993]. Knowledge of this phenotype has since been expanded to include a wide variety of conditions including diabetes mellitus Type I and II [Kadowaki et al., 1994; Odawara et al., 1995], deafness [van den Ouweland et al., 1992; Remes et al., 1993], migraine and other atypical symptoms such as depression [Shanske et al., 1993], GI problems, cardiomyopathy [Dougherty et al., 1994; Sato et al., 1994], and chronic progressive external ophthalmoplegia (CPEO) [Moraes et al., 1993]. The clinical presentation of the "classical" mitochondrial syndromes such as MELAS, MERRF (myoclonus epilepsy and ragged red fibres), and Kearns Sayre syndrome can overlap widely and often the specific diagnosis can only be confirmed on mtDNA analysis. This A3243G mitochondrial mutation affects the tRNA formation and transcription termination [Kobayashi et al., 1991] and results in deficient activities of Complex I and, to a lesser extent, Complex IV of the respiratory chain [Miyabayashi et al., 1993; Kobayashi et al., 1986; Campos et al., 1994].

The phenotype of mitochondrial diseases depends on the "severity" of the mutation, replicative segregation, the degree of heteroplasmy in the cells, tissue or organ, the threshold of expression of that tissue, and the bioenergetic requirements of the tissue and many modifying factors including age [Shoffner and Wallace, 1995] and, no doubt, other genes. Therefore, tissues

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most highly dependent on energy metabolism and ATP production such as the brain, muscle, kidney, pancreas, and liver in decreasing order are at highest risk of expressing disease.

We describe a family demonstrating the extreme clinical variability now known to be associated with the A→G transition at position 3243 of the mtDNA. The structural abnormalities consistent with diabetic embryopathy, detected only in one of a set of twins, suggests that this mitochondrial mutation, in the inner cell mass and the extraembryonic membranes of the twins, predisposed him to the teratogenetic effects of the maternal diabetes also caused by this mutation. The differences in manifestations probably are the result of the distribution of the mutation in the embryonic/fetal and placental tissues.

CASE REPORTS

Case IV-4 (Fig. 1), the propositus, a dizygotic male, twin B, was born to a 31-year-old G3P0SA1TA1L0 (now G3P2L2) mother of Italian descent and a father of Armenian descent. The parents were nonconsanguineous. The mother (III-5) developed type I IDDM at 20 years and now receives Humulin N® 30 units morning dose and 20 units evening dose. At 9 weeks pregnancy her hemoglobin A_1 was 0.065 SI units. There was apparently poor preconceptual diabetic control which was not monitored by a physician. At 22 years, Crohn's disease

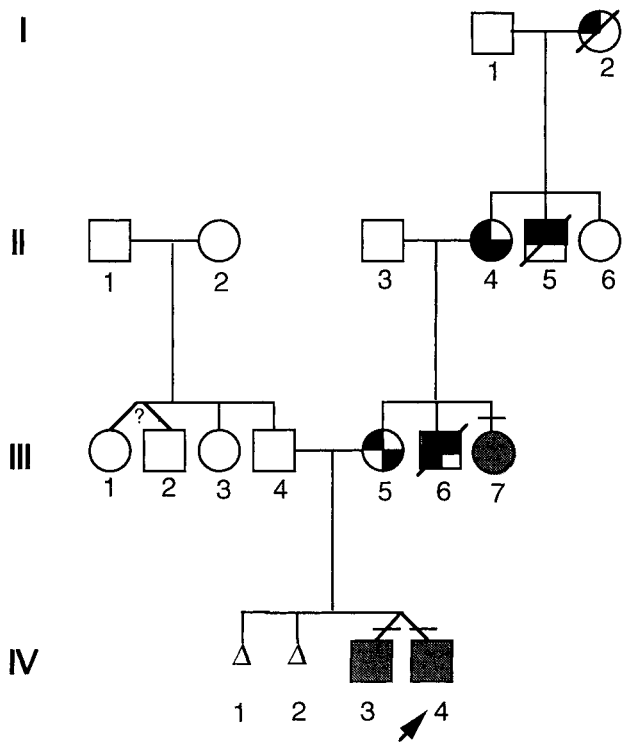

Fig. 1. Pedigree of the family studied. The symbols □ and ○ indicate male and female, respectively. The arrow indicates the propositus. ◩ indicates those affected with diabetes mellitus, ◨ strokes, ◔ gastrointestinal problems, and ◧ deafness. The zygosity of III-1,2 is unknown. In addition, IV-3 had infantile seizures and IV-4 had caudal dysgenesis. ▩ indicates those tested and shown to be carrying the A3243G mtDNA mutation.

was diagnosed. She has had chronic intermittent bouts of diarrhea which respond to treatment with 5-aminosalicylic acid. Histological and radiological examinations have not been performed.

The pregnancy was initiated by medical induction of ovulation and was complicated by diabetes treated with insulin, and hypertension treated with alpha-methyldopa for the duration of the pregnancy. Delivery was by Cesarean section at term and was uncomplicated. Two placentas were examined and normal.

Birth weight was 3.09 kg (50%), length was 49.5 cm (25–50%) and head circumference was 33 cm (10%). Apgar scores were 5 and 7 at 1 and 5 minutes, respectively, and he was noted to be hypotonic. He had anal atresia, a rectourethral fistula, bifid scrotum, discrepancy in size between the larger right and smaller left lower limb, and overriding toes. Skeletal survey showed diminished height of a right L5 hemivertebra and loss of the pedicles of S3 on the right and S2 and S3 on the left with fusion of S4 and S5. There was also bilateral hip "dysplasia." A metaphyseal lucent line was noted within the distal femur and proximal tibia as well as slight central lucency of the distal humerus. There was bone asymmetry in the lower limbs. Ultrasound (U/S) structure of the spinal cord was normal. Head U/S findings were normal. Abdominal U/S showed a left multicystic dysplastic non-functioning kidney with dilatation of the distal end of the left ureter. The right kidney showed multicystic dysplastic changes with dilatation of the pelvicalyceal system but no dilatation of the ureter in keeping with obstructive dysplasia. The renal arteries and veins were patent.

At 3 days of age, he had a colostomy. By age 15 months his anal atresia was corrected with a pull-through procedure and the colostomy was closed. After this operation, he was noted to have rectal prolapse and at 18 months had rectal reconstruction. During the first 2 months of life, his urological problems were initially treated with an indwelling suprapubic catheter, later with right uretero and urethral stent insertion and subsequently a right nephrostomy tube was inserted allowing removal of the stents. The nephrostomy tube was removed at age 2 months. At 8 months he required multiple urethral dilatations due to a urethral stricture secondary to the rectourethral fistula. Progressive renal dysfunction ensued associated with rickets and a fracture of the right distal tibial metaphysis. At 18 months, he had growth failure and developmental delay especially of gross motor development. At 2 years his renal dysfunction had progressed, requiring dialysis.

Analysis of DNA extracted from whole blood at age 2½ years (see Methods) confirmed the presence of the A3243G mtDNA mutation at 43% heteroplasmy.

Maternal uncle (III-6) developed IDDM at age 26 years and was treated with insulin. Six months later, he had a stroke that left him handicapped. He apparently had some hearing problems. A second stroke a year later resulted in further deterioration, dementia and death at the age of 38. Further details are not available.

Maternal aunt (III-7) was 28 years old at the time of assessment at our institute. She had short stature, a height of 145 cm (<3rd centile), dysarthria and a

seizure disorder treated with valproic acid and diphenylhydantoin since age 19 years when an episode of encephalopathy left her with some cognitive dysfunction. At age 26 years she had another episode of encephalopathy with seizures, lateral gaze nystagmus, ptosis, dyskinesia, left hemiparesis, impairment of left lateral gaze, and a visual field defect on the left side. CT brain scan showed a right temporal infarct, an older left frontal infarct and generalised cortical atrophy. There was a small area of probable infarction in the left inferior aspect of the left basal ganglia adjacent to the thalamus and within the posterior limb of the internal capsule. Results of cerebral angiogram and carotid Doppler study were normal. Blood and CSF lactate were both elevated at 4.6 and 8.0 mmol/L(normal <2.5), respectively. EEG showed a diffuse abnormality with theta dominant and asymmetrical activity. Brain biopsy done during the second episode showed extensive astrogliosis involving the cortex and underlying white matter. There was also prominent neuronal loss and spongy change following a laminar distribution. Electron microscopy (EM) showed elongated thin mitochondria with atypical cristae. Temporalis muscle biopsy showed many atrophic fibres. Gomori's modified trichrome staining showed prominent subsarcolemmal red staining and irregular ragged-red-like deposits in a few fibres. EM findings were normal. During the second episode, she was noted to have severe bilateral sensorineural hearing loss and diabetes mellitus. She was treated with insulin and later with oral glyburide with good control. At age 27 years she was assessed for secondary amenorrhea, acne, and hirsutism. Endocrine studies showed normal FSH, LH, cortisol, TSH, and T4 levels but low estradiol 85 pmol/L (normal >110 pmol/L) not responsive to a Provera challenge test. Abdominal u/s findings were normal. She was diagnosed to have androgenization or hypoestrogenism but was not compliant with hormonal therapy. Cardiological findings were normal. Auditory brain stem evoked potentials showed absent responses bilaterally, visual evoked potentials showed delayed responses, and median nerve somatosensory evoked potentials were normal. At 29 years, analysis of mtDNA from peripheral blood leukocytes confirmed the heteroplasmic presence of the $tRNA^{Leu(UUR)}$ A→G mutation at position 3243 (31.7% mutant).

Maternal grandmother (II-4) was 63 years old. She had Type II diabetes, deafness needing hearing aids and chronic "gastrointestinal complaints." Her brother (II-5) was diabetic and died after a stroke at age 54 years. The maternal great grandmother (I-2) was by history also diabetic and died in her thirties of pneumonia.

Case IV-3, the co-twin of the propositus, was the first-born. Birth weight was 2.9 kg (25th centile). Apgar scores were 7 and 9 at 1 and 5 minutes, respectively, and no abnormalities were noted on physical examination. There were no concerns during the first 3 months of life and his growth and development were normal. At 3 months, he developed recurrent generalised clonic seizures associated with lip smacking and eye rolling lasting 15–20 seconds each. Between seizures, he was attentive, reactive and smiling. He was treated with phenobarbital, Mogadon® and Primidone®. Results of investigations including CSF protein, glucose, glycine and lactic acid levels, blood electrolytes, lactate, ammonium, liver function tests, blood gases, biotidinase activity, very long chain fatty acid levels and karyotype, urinary organic acids, amino acids, oligosaccharides, mucopolysaccharides, and S-sulfocysteine analyses, leukocyte hexosaminidase, galactocerebrosidase, β-glucosidase, β-galactosidase, and arylsulfatase A activities, ophthalmological examination and brain MRI were all normal. Scanning EM study on skin biopsy, hair and peripheral leukocytes showed neither intracellular inclusions nor abnormal mitochondria. EEG showed intermittent generalized paroxysmal slow wave activity but no epileptiform activity was noted. Visual evoked potentials were abnormal with delayed low amplitude responses, auditory brainstem evoked potentials (ABRs) were normal but the median nerve somatosensory evoked potential was abnormal with no responses detected.

Following the diagnosis of MELAS in his maternal aunt (III-7), mitochondrial DNA analysis was performed and showed heteroplasmy of the $tRNA^{Leu(UUR)}$ A3243G mutation of the mtDNA in both blood (28% mutant) and cultured fibroblasts.

At the age of 18 months, patient IV-3 weighed 17.6 kg (>97%), was 89 cm (>97%) long, and had an OFC of 50.5 cm (90%). His facial appearance was normal. Neurological examination revealed normal cranial nerves and normal muscle strength and reflexes. His psychomotor development, including gross motor, speech and adaptive skills, was globally delayed and equivalent to that of about a 1 year old.

MATERIALS AND METHODS

DNA from whole blood or cultured fibroblasts were prepared for polymerase chain reaction (PCR) analysis using Chelex 100 resin (Biorad Laboratories, Hercules, CA) as outlined elsewhere [Koo et al., 1993]. Amplification was performed using 20 or 40 μl of the DNA preparations. Forward primer (AGGACAAGAGAAATAAGGCC) 20mer corresponding to nucleotides 3130-3149 of mtDNA (Cambridge sequence) and reverse primer (AGTAGCCCGTAGGGGCCTACC) 21mer corresponding to nucleotides 3443-3423 of mtDNA were used. Amplification was for 30 cycles with annealing, extension and denaturation temperatures of 55, 72, and 94°C respectively for 1 minute each. PCR products were extracted with chloroform and precipitated with ethanol. The presence of the A3243G mutation was detected by analysing the products of this PCR reaction after overnight digestion with the restriction enzyme Apa I. The presence of the mutation creates a new Apa I site in the PCR product which, when cleaved, gives rise to fragments of 180bp and 134bp compared to the wild-type parent product of 314bp [modified from Goto et al., 1990]. Electrophoresis through 2% agarose gels was performed. Percentage heteroplasmy of the analyses on the blood samples was estimated from densitometry of the restriction digested PCR products. Results are reported above for each case described, where applicable.

DISCUSSION

The $tRNA^{leu}$ (A3243G) mitochondrial DNA mutation was originally identified in heteroplasmic form in patients with MELAS syndrome [Goto et al., 1990]. However, further studies have documented a broader clinical spectrum as well as intrafamilial and interfamilial variability in the disease manifestations. Among the known conditions associated with this mutation are diabetes mellitus type I and II, deafness, cardiomyopathy, and CPEO as described in the introduction to this paper.

The family reported by us confirms the clinical findings previously reported in association with this mutation and adds important clinical observations which expands the phenotypic spectrum of this syndrome to include infantile seizures and congenital abnormalities consistent with diabetic embryopathy.

Two members of this family had IDDM and three others had non-insulin dependent diabetes mellitus. In most cases IDDM is an autoimmune disorder which results in destruction of the pancreatic β-cells and is associated with low recurrence risk: 1–3% for offspring of affected mothers and 6.1% for offspring of fathers with IDDM [Warram et al., 1988]. However, recent studies showed that both type I and type II diabetes mellitus may be due to the A3243G mitochondrial mutation [Odawara et al., 1995; Kadowaki et al., 1994]. The incidence of this mutation among patients with familial IDDM was 6% and was found in 60% of the patients with diabetes and deafness in a Japanese population [Kadowaki et al., 1994]. In our family, the mother of the twins (III-5) developed IDDM at the age of 20 and her brother developed IDDM, multiple strokes, and deafness after 28 years of age. Given that the mother (III-5) is an "obligate carrier" of the mutation and that her diabetes and gastrointestinal problems are probably the result of this mutation, it is likely that she has significant heteroplasmy of the mutation and that her twin sons (IV-3 and IV-4) inherited the mutation. She has refused further testing. The percentage heteroplasmy as reported in the blood samples of our cases is not usually a reflection of the higher percentage heteroplasmy usually seen in other tissues and organ systems due to the process of mitotic segregation and tendency towards homoplasmy.

The propositus (IV-4) was born with anal atresia, urogenital and vertebral abnormalities including caudal dysgenesis. To the best of our knowledge this is the second report of embryopathy associated with the $tRNA^{leu}$ A3243G mitochondrial DNA mutation [Damian et al., 1995]. In their case however, maternal diabetes was not present.

The incidence of congenital abnormalities among offspring of mothers with IDDM is 2–4 times higher than the risk in the general population with 10% of the abnormalities being major and accounting for 40% of the perinatal deaths [Rosenn et al., 1994]. Among the abnormalities most commonly found in infants of diabetic mothers are neural tube defects, holoprosencephaly, congenital heart defects, caudal dysgenesis sequence, situs inversus, skeletal anomalies, and urogenital anomalies [Goto and Goldman, 1994]. The caudal dysgenesis sequence occurs 200 times more frequently in infants of diabetic mothers although only 8–16% of these are born to mothers with IDDM [Kučera et al., 1971]. The pattern of congenital abnormalities in the offspring of diabetic mothers reflects an insult occurring during early embryogenesis before the 7th week of gestation.

The pathogenesis of this group of congenital abnormalities is not known and most of the information is based on in-vitro animal studies. The anomalies of the infants of diabetic mothers arise during blastogenesis and may be regarded as an association rather than a morphogenetic malformation as discussed by Opitz [1993]. Among the maternal metabolites suspected of having a major pathogenetic role in diabetic embryopathy are hyperglycemia, hypoglycemia, hyperinsulinemia, somatomedin inhibitor, β-hydroxybutyric acid, pyruvate, and α-ketoisocaproate [Eriksson, 1995]. Hyperglycemia may also induce reduced availability of arachidonic acid. Supplementation with arachidonic acid has been shown to reduce the incidence of all embryonic malformations in offspring of diabetic mothers [Goldman et al., 1985]. The fetuses and embryos carried by mothers with IDDM are in a state of chronic hypoxia. Maternal factors causing the fetal hypoxemia include diabetic vasculopathy, alterations in the oxyhemoglobin dissociation curve, diabetic ketoacidosis, and hyperglycemia. Moreover, uteroplacental blood flow is reduced in mothers with IDDM which contributes to the fetal hypoxia [Garner, 1995]. Fetuses exposed to high glucose levels have difficulties tolerating chronic hypoxia [Myers, 1981] which could result in lactic acidosis and further fetal/embryonal damage. Maternal hyperglycemia and the high levels of the other diabetic metabolites have a major impact on immature embryonic mitochondria. In rats, the embryonic mitochondria of offspring of diabetic mothers showed marked swelling and increased number of cristae on transmission electron microscopy, suggesting oxidative stress [Yang et al., 1995]. The same changes were noted in embryonic mitochondria subjected to a high concentration of β-hydroxybutyrate in vitro. This damage to the immature mitochondria in the embryo or placenta and later in the fetus may result in inhibition of oxidative phosphorylation and lack of production of ATP and other energy-rich cellular compounds which could impair the energy-dependent cellular activities such as cellular proliferation, differentiation and cell migration. Hyperglycaemia may enhance oxidative metabolism and thus cause an increased flow of electrons through the electron transport chain and exaggerated generation of free-oxygen radicals [Horton and Sadler, 1985; Eriksson and Borg, 1991, 1993; Eriksson, 1995]. In vitro studies have shown that when high-glucose cultured embryos were concomitantly subjected to alpha-cyano-4-hydroxycinnamate, which inhibits the mitochondrial uptake of pyruvate, it blocked the expected glucose-induced dysmorphogenesis [Eriksson and Borg, 1993]. Furthermore, transgenic rat embryos, overexpressing the superoxide dismutase gene, were protected from the teratogenic effects of an elevated glucose concentration in vitro [Eriksson et al., 1993].

The importance of the role of hyperglycemia in the pathogenesis of diabetic embryopathy has been debated [Garner, 1995] but is best illustrated by the substantial decrease in the incidence of serious congenital abnormalities among offspring of mothers with IDDM who had tight control of blood glucose levels early in pregnancy [Steel et al., 1990].

The A3243G mitochondrial mutation is known to interfere with oxidative phosphorylation and cause reduced activity of Complex I and IV of the mitochondrial respiratory chain [Miyabayashi et al., 1993; Kobayashi et al., 1986; Campos et al., 1994]. Thus, this mutation could lead to an increased production of free-oxygen radicals which could further increase the risk of the congenital abnormalities in cases where the maternal diabetes is related to the mitochondrial DNA mutation.

In the family reported by us the twins were dichorionic dizygotic and exposed to the same maternal diabetic metabolites. However, the propositus (IV-4) developed malformations while his brother (IV-3) had no structural abnormalities. This unique "experiment of nature" could be the result of a difference in the proportion of the abnormal mitochondrial DNA in the placentae and embryonal cells which made one twin more vulnerable to the toxic effect of the diabetic metabolites and affected the ability of some of his cells to produce sufficient cellular energy for normal proliferation, migration and differentiation.

Our pedigree expands the phenotype associated with the A3243G mutation even further to include intractable generalised tonic-clonic seizures in infancy. Case IV-3, despite having intractable generalised seizures, did not have any of the other neurological features classically associated with this mutation such as myoclonic or focal seizures, elevated CSF lactate, stroke-like episodes, or abnormal MRI brain scan. Interestingly, even in the presence of neurological disease (seizures) caused by a mitochondrial DNA mutation, his ABRs were normal. By the age of 2 years he had been seizure free for 8 months, on treatment, and although developmentally delayed, showed no other symptoms or failure to thrive. In the absence of the maternal family history it is unlikely that the diagnosis of a mitochondrial disease would have been considered so early in life. Two infants have been described to present with failure to thrive at age 2 and 3 months [Koo et al., 1993]. One of them, later, had only focal seizures, and both subsequently developed strokes and lactic acidemia by age 15 months, compatible with MELAS. Furthermore, an infant son of a woman with MELAS who died at age 18 hours of "cardiorespiratory insufficiency" was also found to carry the A3243G mutation [Remes et al., 1993]. These reports indicate that mitochondrial DNA mutations should be sought in cases of infantile seizures or other nonspecific symptoms especially if there is evidence for diseases known to be associated with mitochondrial mutations in the mother and/or her relatives.

Gastrointestinal abnormalities including chronic diarrhoea [Dougherty et al., 1994] have also been reported in association with mitochondrial cytopathy. The mother in our report (III-5), experienced gastrointestinal symptoms, had a good response to 5-aminosalicylic acid treatment with repeated relapses and thus was diagnosed as having "Crohn's disease." This has not been confirmed histologically. These gastrointestinal symptoms may also be a manifestation of the A3243G mutation. It may be interesting to investigate the role of mitochondrial cytopathy in the cause of chronic gastrointestinal disease.

Patient III-7 in our family, has developed features of premature menopause with hypoestrogenism. This finding may be an additional clinical manifestation associated with the A3243G mutation as described in other mitochondrial diseases.

The clinical phenotype associated with the various mutations of mitochondrial DNA will likely continue to expand as clinicians become familiar with the variability in presentation and as the molecular technology needed to diagnose them becomes more widely available. Further studies are needed to determine the incidence of the $tRNA^{Leu(UUR)}$ A3243G mitochondrial mutation among diabetic women who have given birth to newborns/fetuses with congenital abnormalities in order to determine the extent of this association.

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